

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

1-4

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



4/29/04

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/41, 31/70, 38/10, 39/395, C07H 5/06, 21/02, 21/04, C07K 7/08, 16/18, 16/32, G01N 33/48, 33/574	A1	(11) International Publication Number: WO 96/32106 (43) International Publication Date: 17 October 1996 (17.10.96)
(21) International Application Number: PCT/US95/04553 (22) International Filing Date: 11 April 1995 (11.04.95) (71)(72) Applicants and Inventors: BOGOCH, Samuel [CA/US]; 46 East 91st Street, New York, NY 10028 (US). BOGOCH, Elenore, S. [US/US]; 46 East 91st Street, New York, NY 10028 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>

(54) Title: AGLYCO PRODUCTS

(57) Abstract

This invention concerns products and methods for the diagnosis and treatment of disorders of conjugated carbohydrate constituents which contribute to cell dysfunction and cell death. The invention teaches that where normal carbohydrate constituents are covalently bound with other cell structures in the form of glycoconjugates, these carbohydrate constituents contribute to cell stability, to receptor and recognition functions of the cell, and to the protection of cell constituents from damage. When these carbohydrates are reduced in concentration or structurally altered, together defined as aglyco states, the stability, receptor, recognition, and protective functions of these carbohydrates are diminished or lost, and cell dysfunction and death result, with disease states. These disease states, in the nervous system for example, include dementias (as in schizophrenia and brain tumors), Parkinsonism and Alzheimer's Disease. These disorders can be diagnosed 1) by direct determination of structural changes in the nervous system glycoconjugates; or 2) because these aglyco products may act as antigens, by the determination of antibodies produced by the body against the aglyco products. Antibodies produced by the body against aglyco products can have a deleterious effect (e.g. in normal developing brain) or desirable effect (e.g. in brain tumors).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

AGLYCO PRODUCTS:**The Invention**

This invention concerns the discovery of products and methods to aid in the diagnosis and treatment of disorders of conjugated carbohydrate constituents of living organisms which contribute to cell dysfunction and cell death.

The present invention teaches that where normal carbohydrate constituents are covalently bound or otherwise closely associated with other cell structures in the form of glycoconjugates, these carbohydrate constituents can contribute to cell stability, to receptor and recognition functions of the cell, and to the protection of cell constituents from damage by both native and foreign substances. When these normal carbohydrate constituents are reduced in concentration or otherwise structurally altered, together defined here as aglyco states, the stability, receptor, recognition, and protective functions of these carbohydrates are diminished or lost, and cell dysfunction and cell death result, with resultant disease states depending on the location of the cell dysfunction or cell death. Evidence here presented indicates that these disease states, in the nervous system for example, as a result of cell dysfunction or cell death (i.e. cell loss), include but are not limited to the dementias, as seen in schizophrenia (dementia praecox) and brain tumors, and also in Parkinsonism and Alzheimer's Disease. This invention teaches that these disorders can be diagnosed by the products of aglyco pathology as follows: 1) by direct determination of structural changes in the nervous system glycoconjugates which changes produce novel aglyco products; or 2) because these novel aglyco products may act as antigens, by determination of the novel antibody products produced by the body against the novel aglyco antigenic products. Thus these aglyco cell antigen products may be sufficiently different from the normal constituents that the body recognizes the new aglyco products as foreign and makes antibodies against them, which antibodies can have a deleterious or desirable effect on the cell and cause cell death (eg. in normal developing brain, not desirable; in brain tumors, desirable). The identification of these aglyco products by direct determination or by determination of the antibodies raised against them, can be an aid to, or the basis for, diagnosis of the disorder as well as the basis for its treatment.

Many seemingly unrelated observations in the past, which were not understood, and could not be understood in terms of cell dysfunction, cell death, and specific disease states, can now be understood in the light of the present invention

which defines for the first time the state of, and the consequences of, aglyco pathology, its products, as well as products and processes for its detection and treatment.

EXAMPLE 1. Glycoconjugate receptors in brain for influenza virus; 'Glyco decoys'.

One example of the function of glycoconjugates is seen as receptor in the attachment of influenza virus to target cells in the brain which the virus is invading. When the influenza virus approaches the cell, it attaches specifically to a glycoconjugate receptor in the cell membrane, in this case a neuraminic acid containing glycoconjugate, and cleaves the neuraminic acid from this receptor by means of the specific influenza virus enzyme neuraminidase (26,29). In the case of one such receptor, the glycoconjugate of brain ganglioside, the cleavage of neuraminic acid brings the virus in contact with a hexosamine, which is a powerful inducer of the ejection of DNA from the virus, permitting its transfer into brain cells (29).

In defense, cells which are the target of the virus release showers of small molecular weight substances containing neuraminic (sialic) acid, the sialoresponsins (38), which may act as decoys for the virus, preventing its attachment to and entry into the cells, much as modern supersonic jet warplanes release showers of metal particles to act as decoys for the attachment and explosion of missiles which are headed to attach to the metal of or in the plane and destroy it. The concentration of the sialoresponsins produced is related to the hemagglutination concentration of the virus. Any glycoconjugates, or products which contain the neuraminic acid-hexosamine linkage, or any other glycoconjugate or structurally similar elements which are recognized by the virus as its receptor, will therefore be useful to act as preventive or therapeutic decoys in influenza and other virus or other infections which use this or similar configurations as receptor. These aglyco decoys may be administered per os, percutaneously, systemically (eg. intravenously or intraperitoneally) or as spray for nasal or respiratory cavities, and by any other means which can bring the virus and the decoy in contact with each other before the virus infects cells for the first time, or infects additional cells after leaving the primary target cells.

EXAMPLE 2. Influenza virus infection, brain cell loss, and deficient neuraminic acid and hexosamine aglyco products in schizophrenia.

The significance of the cell damage or cell death which may be a consequence of influenza virus infection in the developing brain is seen in this Example which relates influenza virus infection to neuronal cell injury, cell death and thus loss, and to schizophrenia, and demonstrates directly and quantitatively the nature of the aglyco products involved.

Earlier quantitative neurochemical studies suggested that neuraminic acid and hexosamine are part of a glycoconjugate intercellular recognition 'sign-post' system which forms the neural networks underlying normal brain development and behavior. Recent histological studies of schizophrenic brain reveal neuronal disorganization which might result from some developmental injury. Epidemiological studies now suggest that infection prenatally with influenza virus, which contains neuraminidase, predisposes to schizophrenia. That conjugated neuraminic acid and hexosamine in cerebrospinal fluid are quantitatively decreased in schizophrenia is shown here in a double-blind study.

The frequency of schizophrenia appears to be increased, from recent epidemiological observations, in individuals whose mothers had influenza virus infection during the second trimester of the individual's gestation (1-9), and in populations with a high frequency of ganglioside and other glycolipid disorders (10). Histological studies demonstrate neuronal cell loss and neuronal disorganization in schizophrenic brain (11-15). The removal of neuraminic acid from its conjugates by the influenza virus enzyme neuraminidase (sialidase) might be responsible for injury to neuronal cell recognition molecules in the immature brain. The above recent findings in epidemiology and histology are relevant to quantitative neurochemical data which demonstrates a decrease in the actual concentration of neuraminic acid and hexosamine conjugates in cerebrospinal fluid (CSF) of schizophrenic patients (16-23,36,37,39), here confirmed, and the related work on glycoconjugate (glycolipid, glycoprotein) recognition substances in brain (24-33) which led to the 'sign-post' hypothesis of neuronal connectivity and nervous system maturation (34-35).

Work in this laboratory led to the conclusion that glycoconjugates in nervous system are recognition substances (24-35). After the structure of the brain gangliosides was first sufficiently determined, gangliosides *in vitro* were shown to act both as receptors for influenza virus, and as effectors, in stimulating clam heart and smooth muscle. *In vivo*, additional ganglioside administered intracerebrally acted as

a "decoy" and inhibited infection of brain neurones by influenza virus. Human brain glycoproteins were isolated in bulk, separated, and partially characterized in terms of their carbohydrate constituents. Glycoconjugates were then shown to be present in synaptic membranes, to be involved in mammalian neural development, and in training and learning tasks in pigeons. The 'sign-post' function of glycoconjugates in nervous system was postulated with reference to the establishment of neural networks, both during developmental maturation and with experience. Schizophrenic patients were shown to have a significant decrease of conjugated neuraminic acid and hexosamine in CSF compared to other psychotic disorders and normals (16-23). This decrease was quantitatively proportional to the severity of the illness, and increased toward normal with improvement in clinical status (36,37,39).

We here illustrate a double-blind study which demonstrates the decrease in conjugated neuraminic acid and hexosamine in CSF of schizophrenic as compared to non-schizophrenic psychotic patients. Of nineteen patients two patients were excluded because the clinical diagnoses were not known. The remaining 17 were designated "schizophrenic" (N=11) when this diagnostic term was used, and "non-schizophrenic" other psychoses (N=6) when schizophrenia was not the diagnosis. These diagnoses were made by careful clinical work-up including full history and assesment of mental status with the Psychotic Characteristics Scale (39) by research psychiatrists who had completed a minimum of three years of psychiatric residency training, and the diagnoses were confirmed by senior staff psychiatrists.

Lumbar CSF specimens, 5 to 18 ml., coded, and without clinical information, were shipped in dry ice to the laboratory. Each of the specimens was lyophilized, dialyzed against distilled water exhaustively (cellophane pore size approx. MW 12,000 Daltons) at 0 - 5°C to remove free neuraminic acid and free hexose, and the non-dialyzable fraction was quantitatively analyzed in duplicate for conjugated neuraminic acid by the Bial's orcinol and thiobarbituric acid (TBA) methods, conjugated hexosamine, and conjugated hexose, all as previously described (19). After the neurochemical tests for all specimens were completed, the code was broken. Despite the small sample size, conjugated neuraminic acid ($p<.025$), and conjugated hexosamine ($p<.006$), but not conjugated hexose, were statistically significantly lower in concentration in the schizophrenic group by two-tailed t

test. Values of conjugated neuraminic acid in 81.8% of schizophrenics were less than 7.5 µg/ml CSF, and in 83.3% of non-schizophrenics were greater than 7.5 µg/ml CSF (see Figure 1). In addition, in three patients, one non-schizophrenic and two schizophrenic patients, who had repeated CSF drawn weeks apart, reproducibility was obtained in the neurochemical analysis of the randomized blind specimens. Determination of conjugated neuraminic acid by the TBA method was shown not to be reliable (38,39). It should be emphasized that in the present and all previous studies, both in vitro and in vivo, it is only the conjugated and never the free neuraminic acid which is found to be biologically active or decreased in schizophrenia.

Thus, previous neurochemical data led to the normal 'sign-post' hypothesis, but the nature of the pathology of glycoconjugates was not understood. That is, the meaning of a decrease in the concentration of neuraminic acid and hexosamine, as glycoconjugates, was not understood. That a decrease in these glycoconjugates pre- or post-natally, due to virus or other acute or chronic cause, is critical in the pathogenesis of schizophrenia was not realized. Consideration of the epidemiological evidence of the relation of influenza virus infection in the second trimester of pregnancy to frequency of schizophrenia resulting, and histological evidence of brain cell loss and disorganization, taken together with direct evidence of the quantitative decrease in neuraminic acid and hexosamine concentration in glycoconjugates, has led to the present discovery of aglyco pathology.

EXAMPLE 4. Treatments for schizophrenia which are beneficial increase the concentration of glycoconjugates in the nervous system concomitant with patient improvement. Reference 39 describes the increase (+) or decrease (-) in absolute amounts of glycoprotein carbohydrate constituents in the cerebrospinal fluid in 65 instances of improvement, "no change", or worsening in the clinical status of 47 schizophrenic patients during entry into and recovery from a psychotic state treated with electroshock or drugs.

EXAMPLE 5. Administration of a neuraminic acid and hexosamine precursor to schizophrenic patients results in improvement. D-glucosamine HCl, 200 mg per day, was administered per os to chronic hospitalized schizophrenic patients for 30 days. The Psychotic Characteristics rating scale was used, together with overall clinical

observation to detect improvement or lack thereof. Over one half of the patients responded favorably and there were no untoward reactions.

EXAMPLE 6. Increase in brain glycoconjugate hexose and in the incorporation of ^{14}C glucose in pigeons during training.

The brains of pigeons at rest compared to training in a Skinner box were compared for a) the absolute amount of glycoconjugate hexose and b) the incorporation of ^{14}C -glucose into brain glycoconjugates.

The results are as follows:

a) ABSOLUTE CONCENTRATION OF GLYCOCONJUGATE HEXOSE

	<u>Number of</u> <u>Pigeons</u>	<u>Glycoconjugate Hexose</u> <u>mean mg/g wet weight brain</u>
Resting, never trained	21	0.63
Resting, 3 to 11 months		
post-training	11	0.69
Training:		
10 minutes	1	9.0
20 minutes	1	8.8
30 minutes	5	10.0, 9.8, 3.9, 3.5, 2.7
45 minutes	1	3.5
60 minutes	5	7.8, 5.5, 2.4

b) ^{14}C -GLUCOSE INCORPORATION INTO PIGEON BRAIN GLYCOCONJUGATES

Ninety microcuries of $1\text{-}^{14}\text{C}$ glucose was injected intravenously into pigeons prior to a rest or training period, and the pigeons were sacrificed at intervals thereafter. The proteins of brain were then extracted and chromatographed from each pigeon brain and radioactivity determined on a scintillation counter. After exhaustive dialysis of the isolated brain protein groups, total radioactivity of the fraction was determined, then the fraction was subjected to stepwise acid hydrolysis with dialysis to free liberated sugars which were quantitatively determined. These sugar constituents were then separated by thin-layer chromatography, stained spots removed by vacuum suction and, together with blanks, counted. The results were as follows:

<u>Minutes after Injection</u>	<u>Counts/minute</u> <u>total brain glycoconjugates</u>	
	<u>Training</u>	<u>Resting</u>
10 minutes	2,000	-
20 minutes	15,000	-
30 minutes	21,000	1,000
45 minutes	6,000	35,000
60 minutes	1,500	-
120 minutes	-	32,000
25.5 hours	-	10,000

The time-course of incorporation is markedly different in the training state as compared to the resting state. In the training state, maximum incorporation occurs within 30 minutes at which time only one-twentieth as much is incorporated in the resting state.

References

1. E. O. O'Callaghan, P. Sham, N. Takei, R.M. Murray. *Lancet* **337**, 1248-1250 (1991).
2. S. A. Mednick, R. A. Machon, M. O. Huttenen, D. Bonnet. *Arch Gen Psychiatry* **45**, 189-92 (1988).
3. E. O. O'Callaghan, P. C. Sham, N. Takei, G. Murray, G. Glover, E. Hare, R. M. Murray. *Schizophrenia Research* **9**, 138 (1993).
4. R. M. Murray, N. Takei, P. Sham, E. O. O'Callaghan, P. Wright. *Schizophrenia Research* **9**, 137 (1993).
5. P. C. Sham, C. J. MacLean, K. S. Kendler. *Schizophrenia Research* **9**, 139 (1993).
6. N. Takei, P. Sham, E. O. O'Callaghan, G. K. Murray, G. Glover, R. M. Murray. *Schizophrenia Research* **9**, 141 (1993).
7. D. Cotter, N. Takei, C. Larkin, P. Sham, R. M. Murray, M. Farrell, J. Oxford, E. O. O'Callaghan. *Schizophrenia Research* **9**, 147 (1993).
8. J. L. Welham, J. J. McGrath, M. R. Pemberton. *Schizophrenia Research* **9**, 143 (1993).
9. R. E. Kendell, I. W. Kemp. *Arch Gen Psychiatry* **46**, 878-82, 1989.
10. M. Rahav, A. B. Goodman, M. Popper, S. P. Lin. *Am J Psychiatry* **143**(10), 1249-1254 (1986); A. B. Goodman. *Schizophrenia Bulletin*, Accepted for publication.
11. F. E. Bloom. *Arch Gen Psychiatry* **50**, 224-227 (1993).
12. S. Akbarian, W. E. Bunney Jr, S. G. Potkin, S. B. Wigal, J. O. Hagman, C. A. Sandman, E. G. Jones. *Arch Gen. Psychiatry* **50**, 169-177 (1993).
13. S. Akbarian, A. Vinuela, J. J. Kim, S. G. Potkin, W. E. Bunney, E. G. Jones. *Arch Gen Psychiatry* **50**, 178-187 (1993).
14. J. A. Kovelman, A. B. Scheibel. *Biol Psychiatry* **19**, 1601-1621 (1984).
15. A. J. Conrad, A. B. Scheibel. *Schizophrenia Bulletin* **13**(4), 577-587 (1987).
16. S. Bogoch. *Am J Psychiatry* **114**, 172, 1957.

17. *ibid.* *AMA Arch Neurol Psychiat* 8 0,221-224(1958).
18. *ibid.* *Nature* 1 8 4, 1628-29(1959).
19. *ibid.* *J Biol Chem* 2 3 5,16-20 (1960).
20. *ibid.* *Am J Psychiatry* 1 1 6,743-747 (1960).
21. S. Bogoch, K.T. Dussik, P. G. Lever. *AMA Arch Gen Psychiat* 1 , 441-446 (1959).
22. S. Bogoch, K. T. Dussik, C. Fender P. Conran. *Am J Psychiatry* 1 1 7,409-415 (1960).
23. S. Bogoch, W. Sacks, G. Simpson. *Neurology* 1 3,355 (1963).
24. S. Bogoch. *J Am Chem Soc* 7 9,3286 (1957).
25. *ibid.* *Biochem J* 6 8,319-324 (1958).
26. *ibid.* *Virology* 4,458-462 (1957).
27. *ibid.* in *Handbook of Neurochemistry*, Vol 1, A. Lajtha, Ed. (Plenum Press, N.1968).
28. *ibid.* in *Protein Metabolism of the Nervous System*, A. Lajtha, Ed. (Plenum Press, N.Y. 1970) pp.555- 569.
29. S. Bogoch, P. Lynch, A. S. Levine. *Virology* 7 ,161-165 (1959).
30. S. Bogoch, E. S. Bogoch. *Nature* 1 8 3,53 (1959).
31. S. Bogoch, M. K. Paasonen, U. Trendelenburg. *Brit J Pharmacol* 1 8,325-329 (1962).
32. S. Bogoch, P. C. Rajam, P. C. Belval. *Nature* 2 0 4,73-75 (1964).
33. S. Bogoch, P. C. Belval, W. H. Sweet, W. Sacks, G. Korsh. *Protides of Biol Fluids XV*,129-135 (1968).
34. S. Bogoch. *The Biochemistry of Memory; with an Inquiry into the Function of the Brain Mucoids*. Oxford University Press, 1968.
35. *ibid.* in *The Nervous System, Vol 1, National Institutes of Neurological and Communicative Disorders and Stroke*, D.B. Tower, Ed. (Raven Press, N.Y., 1975) pp. 591-600.
36. S. Bogoch, K. T. Dussik, P. Conran. *New England Journal of Medicine* 2 6 4,251-258 (1961).
37. S. Bogoch, P. C. Belval, K. T. Dussik, P. Conran. *Am J Psychiatry* 1 1 9,128-133 (1962).
38. S. Bogoch, P. Evans. *Nature* 1 9 5,180 (1962).
39. R. J. Campbell, S. Bogoch, M. J. Scolaro, P. C. Belval. *Am J Psychiatry* 1 2 3,952-962 (1967).
40. E. Robins, A. B. Croninger, M. K. Smith, A. C. Moody. *Ann NY Acad Sci* 9 6,390-1(1962).
41. G. Christoni, R. Zappoli. *Am J Psychiatry* 1 1 7,246 (1960).
42. N. M. Papadopoulos, J. E. McLane, D. O'Doherty, W. C. Hess. *J Nerv Ment Dis* 1 2 8,450 (1959).
43. P. Sirota, H. Bessler, D. Allalouf, M. Daldetti, H. Levinsky. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 1 2 (1),103-7 (1988).

EXAMPLE 7. Aglyco brain glycoprotein 10B, and its specific antibody, are two aglyco products in brain and other malignancies. The antibody against aglyco 10B, produced in the body, causes cell death.

The evidence given in this Example, Example 7, from a different system,

determinable in both the brain glycoconjugate 10B, and determinable by quantitation of the cytotoxic antibody made by the body against the resultant aglyco product of 10B ('aglyco 10B'), illustrates further the principle and the products demonstrated in the earlier Examples. That the elevation in the absolute concentration of this antibody (anti-aglyco10B, or antimalignin) is observed in other common malignancies of different cell types, that is, cancer of the lung, breast, colon, etc. illustrates that aglyco pathology is a general pathological phenomenon, not just restricted to brain.

When glycoprotein 10B was identified as a normal 250 KD membrane constituent in human brain and shown to be involved in training in pigeon brain^{2,5-10}, one of the controls examined to test for the relationship of normal 10B to cell-cell interaction was brain malignancy. It was hypothesized that in brain glioblastoma, where apparent loss of contact inhibition of cell division was accompanied by unrestrained proliferation, the structure of 10B might be expected to be altered. Indeed, the structure of 10B was found to be markedly altered in brain glioblastoma: the carbohydrate groups were reduced in quantity by approximately 50% and there was a loss of heterogeneity. Where in the normal 10B there were 9 different carbohydrate constituents, in tumor 10B these were reduced to 5 or 6. Furthermore, the protein portion of 10B was overproduced or overexpressed 7 to 10 fold. Astrocytin was the name given to the 10KD peptide cleaved from brain tumor 10B. From glioblastoma cells grown in tissue culture, the equivalent to astrocytin, malignin, was isolated, and it proved to be a very close structural relative of astrocytin¹⁶. Malignin was so-named because in tissue culture the expression of this peptide, and thus its concentration per mg. membrane protein extractable, increased with increased rate of cell division per unit time.

Recognin M was isolated from MCF7 malignant mammary cells. It is a 10 kD cancer polypeptide antigen rich in glutamic and aspartic acids, related to malignin isolated from glial brain tumors (Glu13,Asp9,His2)²¹. An IgM³⁷ auto-antibody against Recognin M, antimalignin, has been isolated from human serum²⁰, produced as mouse monoclonal^{31,33}, produced in human form by challenge of human lymphocytes with the antigen *in vitro*^{35,37}, and has been isolated from malignant cells obtained at surgery and autopsy²⁴ by elution and immunoabsorption to its immobilized purified antigen.

CLINICAL DATA

In a 20-year randomized mostly blind study involving several hundred physicians and three independent laboratories in the U.S., and three hospitals and one laboratory in the U.K. we have now found that the concentration of antimalignin in serum, in ug/ml, 1) of normal healthy non-tumor-bearing humans increases moderately each decade between the third and the seventh, as the risk of cancer increases ($p < .001$; $N = 1,972$)(Figure 2), 2) increases earlier and more markedly in as yet apparently unaffected members of high-risk cancer families ($p < .001$; $N = 1,106$) (Figure 2 and 4), and 3) is markedly increased in concentration in human serum within weeks of the occurrence of malignant transformation to clinical breast cancer, but is not stigmatic (in the sense of invariant) since it returns to normal within 3 months of successful treatment^{39,41,44,45} and remains in the normal range even up to 27 years after successful treatment (Figures 3 and 4) ($p < .001$; false positives and false negatives $< 5\%$ on first determination, $< 1\%$ on repeat determination; $N = 600$).

An example of how antimalignin is not fixed but is modulated is shown in Table II. Readily biopsied cervix provided pathological evidence of transformation from dysplasia to the stage of frank invasive carcinoma; this was accompanied by marked elevation of the concentration of antimalignin. After surgical removal, antimalignin returned to normal within three months. Figures 3 and 4 show that these are statistically significant changes in both directions ($p < .001$).

Quantitative determination of serum antimalignin antibody is therefore of interest for use as a non-invasive biomarker to indicate successful results in breast cancer chemoprevention trials.

In addition, purified antimalignin antibody (MTAG), because of its demonstrated specificity in fluorescent and other chromogen staining of cell membranes in which the epitopes of malignin have become exposed^{16,27,28,30}, is applicable for use alone or as part of a battery of pre-dysplasia or dysplasia-based surrogate endpoint biomarkers in both individual and computerized cytometry.

RELATION TO PATHOLOGICAL PROCESS: ANTIMALIGNIN IS AN INHIBITORY TRANSFORMATION ANTIBODY

Cells which have undergone malignant transformation in humans may take years to, or may never, proliferate to become clinical cancer. If inhibition of

proliferation is an immune process, as has been theorized^{1,3}, and suggested by the increased incidence of cancer in immunodeficiency disorders such as AIDS, there is no direct evidence in human cancer of such an immune process, and the responsible mechanisms are unknown. Antimalignin, in addition to the properties listed above, is quantitatively related to survival in patients^{32,34}. In vitro, antigen-purified human antimalignin binds to cancer cells regardless of cell type, is present in non-saturating amounts on cancer cells removed at surgery or autopsy, is inhibitory to the growth of small cell lung carcinoma cells at picograms (femtomoles as this is an IgM) of antibody per cell (Figure 5), and is cytotoxic to malignant glial cells (Figure 6 j,k and l).

Antimalignin antibody or derivatives thereof, in human form, that contain its immunological specificity, is given subcutaneously or intravenously to animals or man, in amounts of one to ten grams per day and produces destruction of cancer cells.

Quantitative determination for antimalignin antibody: determined with immobilized malignin antigen (TARGET reagent, Brain Research, Inc., Boston). In the preparation of TARGET reagent, as previously described⁴⁴, human glioblastoma cells were grown in 250 ml sterile tissue culture flasks stacked in the horizontal position in a 37°C incubator until a monolayer of cells had covered the wall of the flask, freed from the wall with trypsin, scraped with spatula into a glass beaker, homogenized with a Branson sonifier, dialyzed, concentrated by pervaporation, centrifuged, chromatographed on a Cellex D column with stepwise elution with buffered solutions of decreasing pH, with the protein in each eluate quantified by adsorption at 280 mμ. The last eluate, which contains malignin, eluted at its pK of approximately 2.7, was rechromatographed. The final preparation contained the malignin with the following composition: Glu13 Asp9 Thr5 Ser5 Pro4 Gly6 Ala7 Val6 Met2 Ileu4 Leu8 Tyr3 Phe3 His2 Lys6 Arg51/2Cys1, and demonstrating a molecular weight of approximately 10K, and dimers and trimers thereof, on SDS gel and thin layer gel chromatography. Malignin was combined covalently with bromoacetylcellulose to produce immobilized TARGET reagent. To quantify the antimalignin antibody in cancer and normal sera, 0.2 ml samples of each serum specimen, in duplicate, were exposed to TARGET reagent with shaking for either two hours or 10 minutes, the bound antibody washed three times with cold NaCl, then released from the antigen by incubation with shaking with 0.25 molar acetic acid, centrifuged at 3000 rpm, the clear acetic acid supernatant read spectrophotometrically at O.D. 280, and the results converted to micrograms of protein. Results are given, as mean +/- S.D. for each serum, for NET TAG (=slow-binding antibody (2 hour contact of eluant with immobilized malignin) minus fast-binding antibody (10 minute contact of eluant with immobilized malignin), microgram/ml serum⁴⁴.

References:

1. Thomas L: Discussion paper P.B. Medawar, in *Cellular and Humoral Aspects of Hypersensitivity*, ed. H.S. Lawrence, Hober - Harper N.Y., 1959, p529
2. Bogoch S: *The Biochemistry of Memory: with an inquiry into the function of the brain mucoids*, Oxford University Press, 1968.
3. Burnet M: *Cellular Immunology*, Melbourne University Press, Cambridge University Press, 1969, pp252-28,.
4. Bogoch S: Inverse Relationship Between Cell Replication and Macromolecular Carbohydrate Concentration: A Distance Factor (DF) in Brain Tumors. Abstr. 3rd Intl meeting of the Intl Soc. for Neurochemistry, July, 1971.
5. Bogoch S: Brain Glycoprotein 10B: Further Evidence of the "Sign-Post" Role of Brain Glycoproteins in Cell Recognition, Its Change in Brain Tumor, and the Presence of a "Distance Factor". Chap P. Mandel, and I.G. Morgan. Plenum Press, 1972, pp. 39-52.
6. Bogoch S: Brain Glycoproteins and Inter-Cell Recognition: Tay-Sachs' Disease and Interneuronal Recognition. Chapter In *Sphingolipids, Sphingolipidoses and Allied Disorders*, Eds. B.W. Volk and S.M. Aronson. Plenum Press, 1972, pp. 127-149.
7. Bogoch S: Astrocytin: Purified Immunologically Active Component of Brain Glycoprotein 10B. Abstracts of 4th Intl. Mtg., Intl. Soc. for Neurochemistry, Tokyo, 1973.
8. Bogoch S: Brain Glycoproteins and Learning: New Studies Supporting the "Sign-Post" Theory. Abstracts of 4th Intl Mtg. Intl. Soc. for Neurochemistry, Tokyo, 1973.
9. Bogoch S: Brain Glycoproteins and Learning: New Studies supporting the "Sign-Post" Theory. Ch. *Current Biochemical Approaches to Learning and Memory*. Eds. W.B. Essman and S. Nakajima. Spectrum Publications, 1973.
10. Bogoch S: Glycoproteins and Brain Circuitry: the "Sign-Post" Theory in Normal Memory Function, and the Regressive States of Brain Tumors and in the Psychoses. Chapter in *Biological Diagnosis of Brain Disorders*. Ed. S. Bogoch. Spectrum-Halstead-Wiley, New York, 1974.
11. Bogoch S: The Detection of malignant Gliomas in Brain by the Quantitative Production In Vitro of TAG (Target-Attaching-Globulins) from Human Serum. Chapter in *Biological Diagnosis of Brain Disorders*. Ed. S. Bogoch. Spectrum-Halstead-Wiley, New York, 1974.
12. Bogoch S: The "Sign-Post" Function of Brain Glycoproteins: Order and Disorder. In *The Nervous System. 25th Anniversary Volume, National Institutes of Neurological and Communicative Disorders and Stroke: Vol. 1, The Basic Neurosciences*. Ed. D.B. Tower. Raven Press, 1975, pp 591-600.
13. Bogoch S: Malignin: A Polypeptide Related to Brain Malignancy. *Transactions of the American Society for Neurochemistry*, 7 (1), 239, 1976.
14. Bogoch S: Brain Glycoproteins and Recognition Functions: Recognins and Cancer. In *Current Trends in Sphingolipidoses and Allied Disorders*. Eds. BL Volk and L Schneck. Plenum Press, New York, 1976. pp. 555-566.
15. Kornblith PL, Caswell PA, Bogoch S, Callahan LV, and Dreyfus J: Effects of Diphenylhydantoin on Cultured Human Glial Cells. *In Vitro*, April 1976, p324.

16. Bogoch S: Astrocytin and Malignin: Two Polypeptide Fragments (Recognins) Related to Brain Tumor. *Natnl. Cancer Institute Monograph* 46:133-137, 1977.
17. Bogoch S: Malignin: Cancer Polypeptide. In *Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain*. Eds. S Roberts, A Lajtha and WH Gispen. Elsevier Press, Amsterdam and New York, 1977, pp 433-440.
18. Bogoch S: Malignin: Polypeptide Cancer Recognin. *Abstracts of the International Society for Neurochemistry Annual Meeting*, Copenhagen, 1977.
19. Bogoch S: Recognins and their Chemoreciprocals. In *Behavioral Neurochemistry*. Eds. J.M.R. Delgado and F.V. DeFeudis. Spectrum-Wiley Press, New York, 1977, pp 197-222.
20. Harris JH, Gohara A and Bogoch S: New Immunodiagnostic Techniques for CNS Tumors. *J. Neuropath. Exper. Neurol.* 3 7:623, 1978.
21. Bogoch S and Bogoch E: New Cancer Recognins, L and M, Immunochemically Related to Brain Malignin. *Transactions of the American ociety for Neurochemistry* 10(1):78, 1979.
22. Bogoch S, Bogoch E, Fager CA, Goldensohn ES, Harris JH, Hickok DF, Lowden JA, Lux WE, Ransohoff J and Walker MD: Elevated Serum Anti-Malignin Antibody in Glioma and Other Cancer Patients: A Seven-Hospital Study. *Neurology* 29: 584, 1979.
23. Bogoch S and Bogoch ES: Disarmed Anti-Malignin Antibody in Human Cancer. *The Lancet* 1:987, 1979.
24. Bogoch S and Bogoch ES: Production of Two Recognins Related to Malignin: Recognin M from Mammary MCF-7 Carcinoma Cells and Recognin L from Lymphoma P3J Cells. *Neurochemical Research* 4: 467-473, 1979.
25. Bogoch S: Cancer Diagnostic Reagents. British patent 1532803, 1979.
26. Bogoch S and Bogoch ES: Tumor Markers: Malignin and Related Recognins Associated with Malignancy Rather than with Cell Type. In *Neurochemistry and Clinical Neurology*, Eds. L Battistin, G Hashim, A Lajtha, Alan R. Liss, Inc. New York 1980, pp 407-424.
27. Redmond FA: Antimalignin Antibody. Doctoral Thesis, Medical College of Ohio at Toledo, 1980.
28. Harris JH, Gohara A, Redmond F, Bogoch S and Bogoch ES: Immunofluorescent and Serologic Studies with Anti-Malignin Antibody. Chapter in *Perspectives of Immunology* .Ed. SA Rosenberg, Academic Press, New York, 1980, pp 571-582.
29. Bogoch S and Bogoch ES: Quantitative determination of Anti-Malignin Antibody. Chapter in *Perspectives in Immunology* .Ed. S.A. Rosenberg. Academic Press, New York, 1980, pp 693- 696.
30. Redmond FA, Harris JH, Loeb TL, Bogoch S, Bogoch ES and Gohara A: Studies of Human Tumors with Anti-Malignin Antibody (MTAG). *FASEB Ann.Mtg. Abstract* no. 40139, 1980.
31. Bogoch S, Bogoch ES and Tsung Y-K: Monoclonal Anti-Malignin Antibodies. *The Lancet* 1:141- 142, 1981.
32. Bogoch S, Bogoch E, Fager CA, Harris JH, Ambrus JL, Lux WE and Ransohoff JA: Detemination of Anti-Malignin Antibody and Malignin in 1,026 Cancer patients and Controls: Relation of Antibody to Survival. *J. Medicine* 13: 22-42, 1982.
33. Bogoch S and Bogoch ES: Malignin, Anti-Malignin Antibody and Scantag. *Protides of Biol.*

Fluids, Pergamon, N.Y. 29: 337-352, 1983.

34. Bogoch S, Bogoch ES, Antich P, Dungan SM, Harris JH, Ambrus JL and Powers N: Elevated Levels of Anti-Malignin Antibody are Quantitatively Related to Longer Survival in Cancer Patients. *Protides of Biol. Fluids*, Pergamon, N.Y. 31: 739-747, 1984.
35. Bogoch S, Bogoch ES and Iliescu VM: In Vitro Production of the General Transformation Antibody Related to Survival in Human Cancer Patients: Anti-Malignin Antibody (AMA). *Cancer Detection and Prevention* 8:033, 1985.
36. Bogoch S and Bogoch ES: Increased Accuracy of Anti-Malignin Antibody Determination in Unstored Sera Permits Screening. *Cancer Detection and Prevention* 11:100, 1987.
37. Bogoch S, Bogoch ES and Iliescu VM: In Vitro Production of the general Transformation Antibody Related to Survival in Human Cancer Patients: Antimalignin Antibody. *Cancer Detection and Prevention* 12:313-320, 1988.
38. Thornthwaite JT, Derhagopian R and Reimer W: Antimalignin Antibody Determination in Patients with Suspicious Mammograms. *Proc Annu Meet Am Assoc Cancer Res* 31, A1550, 1990.
39. Bogoch S and Bogoch ES: Malignin antibody and early malignancy. *The Lancet* 337:977, 1991.
40. Boone CW, Kelloff GJ and Steele VE: Natural History of Intraepithelial Neoplasia in Humans with Implications for Cancer Chemoprevention Strategy. *Cancer Research* 52:1651-1659, 1992.
41. Bogoch S and Bogoch ES: Malignin antibody Returns to Normal After Successful Treatment of Breast Cancer. *Cancer Detection and Prevention* 17(1): 180, 1993.
42. Bogoch ES and Bogoch S: Malignin Antibody As Early Warning. *Cancer Detection and Prevention* 17(1): 229, 1993.
43. Boone CW and Kelloff GJ: Intraepithelial Neoplasia Surrogate Endpoint Biomarkers and Cancer Chemoprevention. *J. Cell Biochem* 17F(Suppl): 37-48, 1993.
44. Abrams MB, Bednarek K., Bogoch S, Bogoch ES, Dardik HJ, Dowden R, Fox SC, Goins EE, Goodfried G, Herman RA, Imperio J, Jackson W, Keuer S, Killackey M, Kimel G, Layton RE, Liebentritt AH, Marsden, McCabe JL, Menasha M, Orten K, Pasmantier M, Pillai T, Pillai VB, Probst W, Reimer W, Smith S, Thornthwaite J, Turner WJ and Whitlock RT: Early Detection and Monitoring of Cancer in Routine Practice with the Antimalignin Antibody Test. *Cancer Detection and Prevention*, 1994;18(1): 65-78.
45. Thornthwaite JT, Derhagopian R and Reimer W: Determination of Antimalignin in Patients with Suspicious Mammograms. *Cancer*, Submitted for Publication 1993.

EXAMPLE 8. The carbohydrate constituents of brain glycoconjugates, reduced in concentration in the presence of brain tumors, can be increased towards normal concentration by certain drugs, eg. Diphenylhydantoin (DPH).

Mice were divided into two groups, one-half which were inoculated with subcutaneous brain ependymomas, and one half which were not. Each of these two groups was divided into two groups: one-half would receive daily

subcutaneous DPH, 1 mg/kg body weight, and one-half which received no DPH but saline injections only. Animals were sacrificed when the ependymoma grew sufficiently just to break the skin surface. There was a significant reduction in the growth of tumors when DPH was given. The brains were pooled for each subgroup, and extracted for protein-bound hexose as in Example 6, with the following results:

<u>Tumor</u>	<u>Brain Protein-Bound Hexose, as % of Protein</u>	
	<u>Without DPH</u>	<u>With DPH</u>
Absent	4.6, 5.5	7.9, 8.0
Present	2.6, 2.3	6.8, 7.0

It should be noted that DPH produces an increase in the concentration of brain protein-bound hexose in the absence of tumors as well as in their presence.

EXAMPLE 9. Structure of Aglyco10B

A. Iodobenzoic Acid Hydrolysis of Aglyco10B Followed by Edman Degradation Yielded the Peptide ("Iodopeptide") with the following sequence:

SEQ ID NO:1 Tyr Lys Ala Gly Val Ala Phe Leu His Lys Lys Asn Asp Ile Asp Glu
Amino Acid

Residue Numbers:1 5 10 15

B. Mass spectrometry followed by calculations using MacBioSpec Software Manual 013048-A, PESCIEX, Perkin Elmer Sciex Instruments, yielded the following properties of the peptide SEQ ID NO:1 :

N-Terminal Group: Hydrogen	C-Terminal Group: Free acid
MH+ Monoisotopic Mass = 1847.9656 amu	HPLC index = 43.40
MH+ Average Mass = 1849.0998 amu	Bull & Breese value = -80
Isoelectric Point (pI) = 8.0	Elemental Composition: C ₈₄ H ₁₃₁ N ₂₂ O ₂₅

C. Mass spectrometry: The sequence of SEQ ID NO:1 in 9A. above obtained by Iodobenzoic acid hydrolysis was independently confirmed by mass spectrometry of fragments of Aglyco10B obtained by four different acid hydrolyses of Aglyco10B in solution and of Aglyco10B immobilized on bromoacetyl-cellulose (Aglyco10BC; or A10BC) as below. These hydrolyses produced 13 overlapping hydrolytic fragments (two of which, 6-10 and 6-12 below, were each obtained by two different hydrolytic methods). Taken together, these overlapping fragments independently confirmed the peptide sequence of SEQ ID NO:1 as follows:

* Intact Aglyco10B in solution is a strong acid, with an isoelectric point of approximately 2.7, and produces autohydrolysis when left at room temperature for hours, or even at 0-5°C for longer periods.

SEQ ID NO:2 Gly Leu Ser Asp Gly Ser Asn Thr Glu Ser Asp Ile
Amino Acid Residue Numbers: 1 5 10

N-Terminal Group: Hydrogen **C-Terminal Group:** Free acid

MH+ Average Mass= 1195.1817 amu Bull & Breese value = 2690

Isoelectric Point (pI) = 4.4

Elemental Composition: C₄₆ H₇₆ N₁₃ O₂₄

E. Mass spectrometry: The sequence of amino acids 2-11 of SEQ ID NO:2 in 2C. above obtained by trypsin hydrolysis was independently confirmed by mass spectrometry of fragments of Aglyco10B obtained by four different acid hydrolyses of Aglyco10B in solution and of Aglyco10B immobilized on bromoacetyl-cellulose (Aglyco 10B-cellulose; or A10BC) as below. These hydrolyses produced 7 overlapping hydrolytic fragments which independently confirmed 2-11 of the peptide sequence of SEQ ID NO:2 Gly Leu Ser Asp Gly Ser Asn Thr Glu Ser Asp Ile as follows:

<u>Fragment Identified:</u> amino acid residue numbers)	<u>MH⁺</u> (mass)	<u>Sequence</u>	<u>Method By Which Fragment Obtained</u>			
			<u>Aglyco10B</u>	<u>A10BC</u>	<u>A10B</u>	<u>A10BC</u>
			<u>Auto-</u>	<u>Auto-</u>	<u>Microwaved</u>	<u>Microwaved</u>
			<u>hydrolysis*</u>	<u>hydrolysis</u>	<u>5 seconds</u>	<u>30 seconds</u>
2-7	592.26	Leu Ser Asp Gly Ser Asn				+
3-5	278.10	Ser Asp Gly				+
4-6	278.10	Asp Gly Ser				+
4-11	824.29	Asp Gly Ser Asn Thr Glu Ser Asp				+
5-8	378.16	Gly Ser Asn Thr	+			
6-10	537.22	Ser Asn Thr Glu Ser				+
8-10	336.14	Thr Glu Ser				+

* Intact Aglyco10B in solution is a strong acid, with an isoelectric point of approximately 2.7, and produces autohydrolysis when left at room temperature for hours, or even at 0-5°C for longer periods of weeks to months.

F. CNBr hydrolysis of Aglyco10B Followed by Edman Degradation Yielded the Dipeptide SEQ ID NO:3 with the following sequence:

Met Asp

Amino Acid Residue Numbers:

1

G. USE OF AGLYCO10B, A10BC, INTACT 10B, SEQ ID NO:1 OR SEQ ID NO 2 AS A VACCINE. Approximately 1mg of any of the above glycoprotein or protein fragments, injected twice or three times subcutaneously in animals including man, produce antimalignin antibody and cellular response for the prevention or treatment of cancer.

-

-

SEQUENCE LISTING:

- (1) GENERAL INFORMATION PATENT APPLICATION
- (i) APPLICANT: SAMUEL BOGOCH
ELENORE S. BOGOCH
 - (ii) TITLE OF INVENTION: AGLYCO PRODUCTS
 - (iii) NUMBER OF SEQUENCES: 2
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: SAMUEL BOGOCH
 - (B) STREET: 46 EAST 91ST STREET
 - (C) CITY: NEW YORK
 - (D) STATE: NEW YORK
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 10028
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: FLOPPY DISK
 - (B) COMPUTER: MAC
 - (C) OPERATING SYSTEM: MAC CLARIS XTND
 - (D) SOFTWARE: MACWRITE
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (vii) PRIOR APPLICATION DATA: N/A
 - (viii) ATTORNEY/AGENT INFORMATION: N/A
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212-831-3070
 - (B) TELEFAX: 212-831-6645
 - (C) TELEX: -
- (2) INFORMATION FOR SEQUENCE ID NO 1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16
 - (B) TYPE: AMINO ACIDS
 - (C) STRANDEDNESS: -
 - (D) TOPOLOGY: UNKNOWN
 - (ii) MOLECULE TYPE

- (A) DESCRIPTION: PEPTIDE
- (iii) HYPOTHETICAL: -
- (iv) ANTI-SENSE: -
- (v) FRAGMENT TYPE: -
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: HUMAN BRAIN GLIOMA,
SUBCULTURE
 - (B) STRAIN: N/A
 - (C) INDIVIDUAL ISOLATE: N/A
 - (D) DEVELOPMENTAL STAGE: UNKNOWN
 - (E) HAPLOTYPE: -
 - (F) TISSUE TYPE: HUMAN BRAIN GLIOMA,
SUBCULTURE
 - (G) CELL TYPE: GLIOMA
 - (H) CELL LINE: GLIOMA
 - (I) ORGANELLE: N/A
- (vii) IMMEDIATE SOURCE: 1) AGLYCO10B, ISOLATED FROM
GLIOMA;
2) AGLYCO10B, IMMOBILIZED ON

BROMOACETYLCELLULOSE.

- (A) LIBRARY: N/A
- (B) CLONE: N/A
- (viii) N/A
- (ix) N/A
- (x) N/A
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
Tyr Lys Ala Gly Val Ala Phe Leu His Lys Lys Asn Asp Ile Asp Glu
1 5 10 15

(3) INFORMATION FOR SEQ ID NO 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12
 - (B) TYPE: AMINO ACIDS
 - (C) STRANDEDNESS: -
 - (D) TOPOLOGY: UNKNOWN

- (ii) MOLECULE TYPE
 - (A) DESCRIPTION: PEPTIDE
- (iii) HYPOTHETICAL: -
- (iv) ANTI-SENSE: -
- (v) FRAGMENT TYPE: -
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: HUMAN BRAIN GLIOMA,
SUBCULTURE
 - (B) STRAIN: N/A
 - (C) INDIVIDUAL ISOLATE: N/A
 - (D) DEVELOPMENTAL STAGE: UNKNOWN
 - (E) HAPLOTYPE: -
 - (F) TISSUE TYPE: HUMAN BRAIN GLIOMA,
SUBCULTURE
 - (G) CELL TYPE: GLIOMA
 - (H) CELL LINE: GLIOMA
 - (I) ORGANELLE: N/A
- (vii) IMMEDIATE SOURCE: 1) AGLYCO10B, ISOLATED FROM
GLIOMA;
2) AGLYCO10B, IMMOBILIZED ON
BROMOACETYLCELLULOSE.
 - (A) LIBRARY: N/A
 - (B) CLONE: N/A
- (viii) N/A
- (ix) N/A (x) N/A
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly	Leu	Ser	Asp	Gly	Ser	Asn	Thr	Glu	Ser	Asp	Ile
1				5					10		

G. A chromophore group ("Chromophore") is present in Aglyco10B which results in Aglyco10B being slightly yellow in a concentrated solution (>100 ug/ml). The Chromophore remains with Aglyco10B throughout all phases of its purification including high pressure liquid chromatography.

When oxygen is replaced by nitrogen by evacuating and heat-sealing the glass tube which contains the Aglyco10B solution, the Aglyco10B solution turns dark green.

When the glass tube is opened to the air, the solution immediately returns to the yellow color.

Since this transition from yellow in the oxygenated state to dark green in the anoxic state and back to yellow in the oxygenated state is a reversible transition seen only in relation to the presence or absence of oxygen, it is concluded that the color change is the property of reversible oxidation:reduction of the Chromophore.

H. Cloning the gene for Aglyco10B. By methods well known in the art, the gene for Aglyco10B and thus for 10B as well, with full amino acid sequence, is derived using either the peptide SEQ ID NO:1 of Example 9A above or the peptide SEQ ID NO:2 of Example 9C above, or both, to construct RNA, and a cDNA probe of normal and transformed glial cell libraries. The gene for Aglyco10B and of 10B permits their use in their entirety, or with fragments of the structure, as vaccines. In addition, the entire gene or parts thereof can be introduced into various well known expression systems to produce intact 10B glycoconjugated protein or Aglyco10B, and these products used as vaccines in the patients, or in animals to produce the specific antibodies to the intact molecules or fragments thereof. Since 10B is known to be involved in recognition and cognitive training in the whole animal, it can be administered to improve cognitive function in animals and man (see Alzheimer's Disease below).

10. Aglyco products in Alzheimer's Disease.

Alzheimer's Disease is a devastating wasting disease in which nerve cell loss is manifested by cognitive disturbances, predominately memory loss in early phases. Certain derivatives of the Amyloid Precursor Protein (APP) are responsible for the abnormal brain deposits characteristic of this disease. Although APP is known to be a glycoprotein, no attention as yet has been paid to the carbohydrates in APP. Because of this invention, aglyco products can be detected 1) directly by isolation and characterization of the derivatives of APP in cerebrospinal fluid, which have decreased carbohydrate components; and 2) indirectly, by detection and quantification in patient's serum of antibodies to Aglyco APP.

Early pre-amyloid deposits in brain have been visualized before the neurofibrillary tangles, the products of nerve cell destruction, have appeared and joined the 'mature' Alzheimer plaque. These pre-amyloid deposits, like the mature plaques, contain Ab peptides, 39-42 amino acid residues long degradation products of APP. While the transition from pre-amyloid to mature

amyloid plaques is likely, since pre-amyloid deposits are present years before degenerating neurites appear in Down's syndrome patients' brain. Pre-amyloid deposits can be more amenable to treatment, that is to dissolution and excretion, before damage to nerve cells occurs. Protection against further accumulation of plaques can be afforded by administering diphenylhydantoin (DPH) which this invention demonstrates increases the concentration of protein-bound hexose in brain (see Example 8 above).

Examples of other conditions in which new aglyco antigens may be exposed and give rise to aglyco antibodies are in Parkinson's Disease and multiple sclerosis, and this invention should be applicable to these and other disorders, both of brain and other organs, both with regard to diagnosis and treatment as above.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
84

What is claimed is:

1. Glyco decoys, being glycoconjugate substances which act as artificial receptors for viruses and other pathogens which would normally attach to and enter the body's cells.
2. Methods for the production and detection of aglyco products being glycoconjugate products with reduced or altered carbohydrate constituents and the aglyco antibodies produced against these aglyco products.
3. Glycoconjugate products which have reduced or altered carbohydrate constituents resulting in the exposure of new antigenic groups or epitopes, such epitopes resulting in the production by the body of anti-aglyco antibodies which in turn injure or destroy the cells in which the aglyco antigen products occur.
4. Aglyco products which occur in schizophrenia and other nervous system disorders, where the aglyco products are deficient in the glycoconjugate's normal complement of neuraminic acid, hexosamine and hexose.
5. Methods to treat schizophrenia and other nervous system and other non-nervous system disorders which result in the increase of glycoconjugate neuraminic acid, hexosamine and/or hexose.
6. Products and methods according to claim 5. whereby hexoses, D-glucosamine and related products and diphenylhydantoin and related products administered to humans results in the increase of glycoconjugate neuraminic acid, hexosamine and/or hexose.
7. Glycoconjugate products which when administered to humans results in improvement in memory and other cognitive functions.
8. Products according to claim 7. which are related to 10B, to the RNA which produces 10B, or to the gene or DNA which controls 10B production.
9. Peptide fragments of 10B or of Aglyco10B, or derivatives thereof, useful in diagnosis and therapy and as a vaccine for the prevention or treatment of cancer.
10. A peptide according to claim 9 where the sequence of the amino acids is tyrosine-lysine-alanine-glycine-valine-alanine-phenylalanine-leucine-histidine-lysine-lysine-asparagine-isoleucine-asparagine-glutamic acid.
11. A peptide according to claim 9 where the sequence of the amino acids is glycine-leucine-serine-aspartic acid-glycine-serine-asparagine-threonine-glutamic acid-serine-aspartic acid-isoleucine.
12. A diagnostic test which by measuring quantitatively antimalignin antibody in serum in cancer establishes that remission of cancer has occurred when the

13. A treatment for cancer consisting of the use of antimalignin antibody, or derivatives thereof, to kill or inhibit the growth of cancer cells.

.....

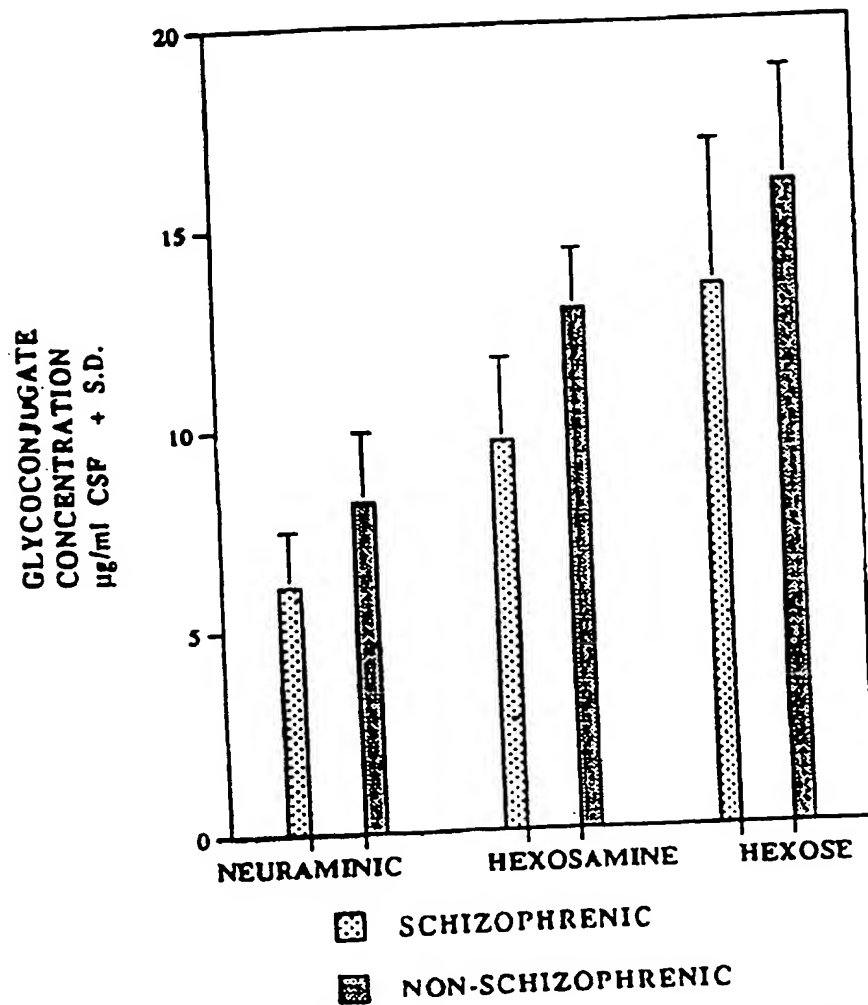


FIGURE 1:

Legend for Figure 1- Glycoconjugate concentration in cerebrospinal fluid in schizophrenia.

2/6

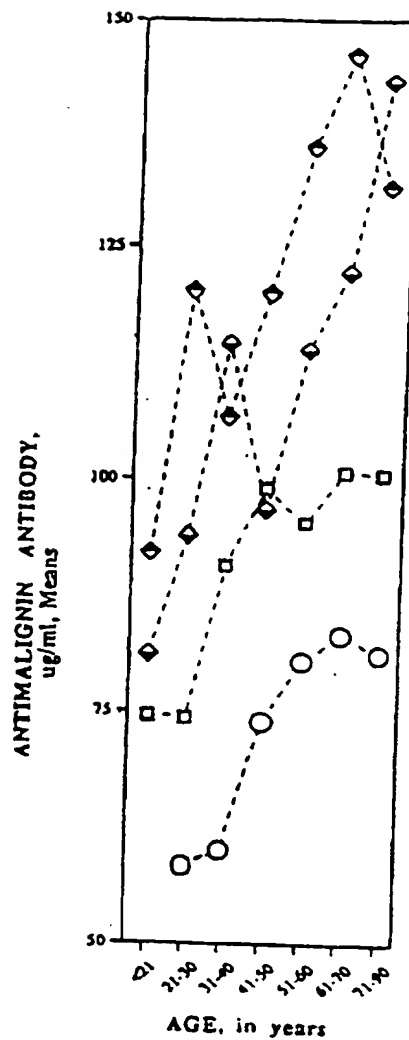


FIGURE 2

Legend for Figure 2- Increase in Antimalignin with age in healthy non-tumor individuals; and the effect of a high frequency cancer family history.

		p compared to --○--
--□--	Screen: Unknown Family History	.332 <.001
--○--	Normal Healthy Controls	1.972
--◇--	Screen: +ve Family History, Asymptomatic	193 <.001
--◇--	Screen: +ve Family History, Symptomatic	181 <.001
TOTAL N =		3,878

SUBSTITUTE SHEET (RULE 26)

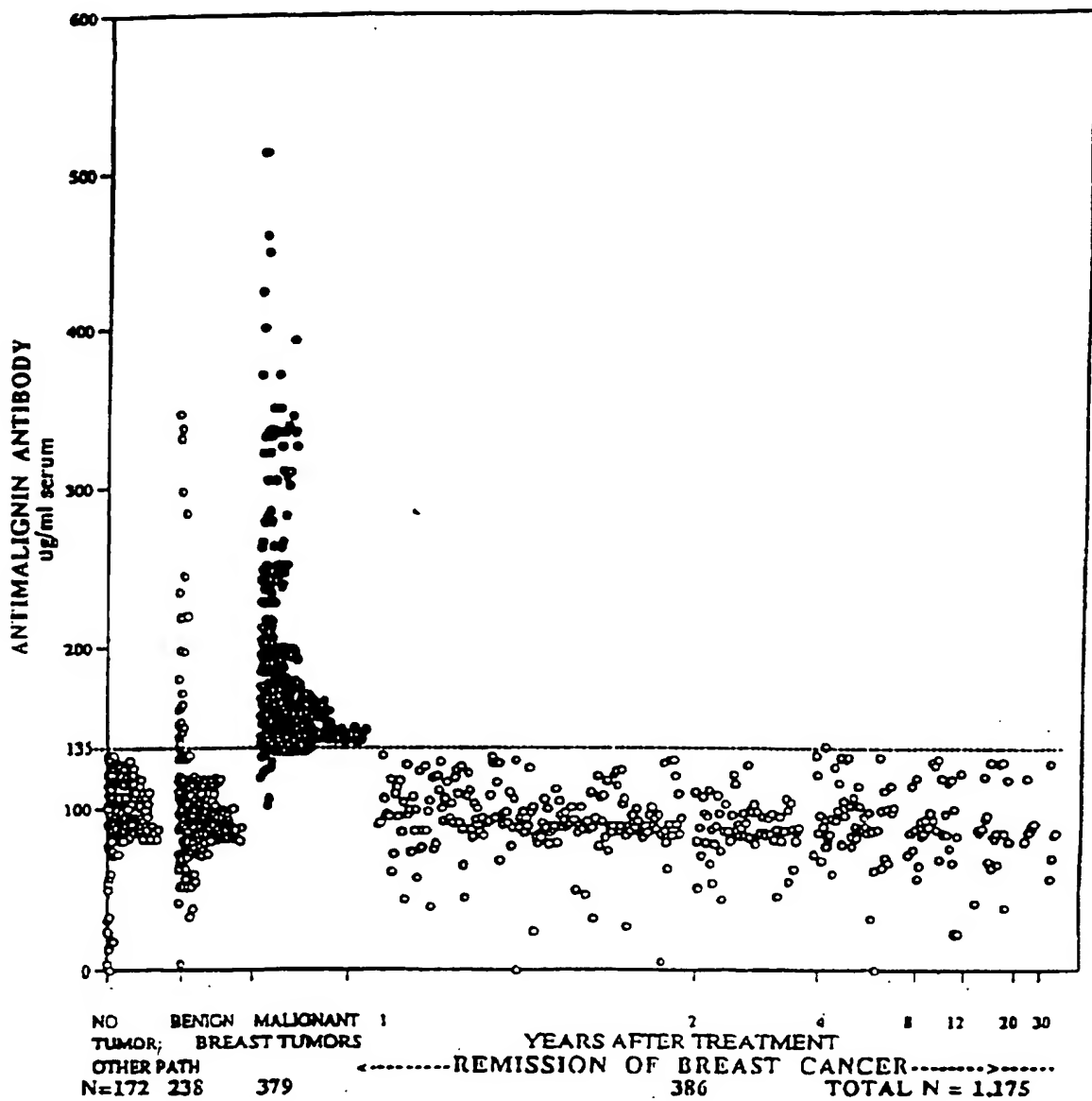


Figure 3

Legend for Figure 3- Antimalignin antibody concentration indicates remission of breast cancer.

4/6

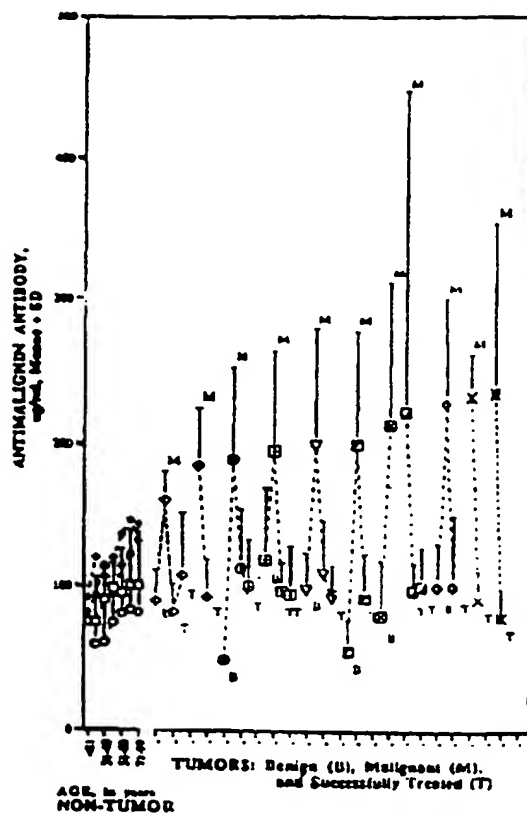
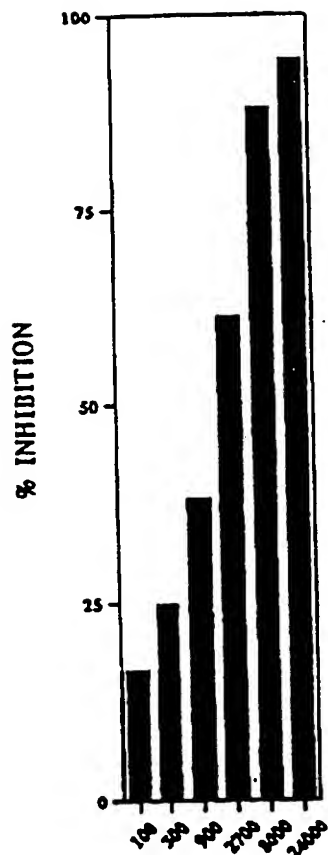


FIGURE 4:

Legend for Figure 4- Antimalignin antibody in non-tumor and tumor populations.

NON-TUMOR		N
--○--	Normal Healthy Controls	1,972
--□--	Screen: Unknown Family History	732
--◇--	Screen: +ve Family History, Asymptomatic	193
--◇--	Screen: +ve Family History, Symptomatic	181
TUMOR		
--◇--	Ovary	58
--◇--	Melanoma	20
--⊕--	Colo-rectal	99
--⊞--	Breast	600
--▽--	Prostate	80
--□--	Conjunctivary	47
--⊞--	Brain	104
--□--	Lung	62
--◇--	Uterus	46
--X--	Basal Cell, Skin	11
--*--	Lymphoma-Leukemia	73
TOTAL N		4,278

SUBSTITUTE SHEET (RULE 26)



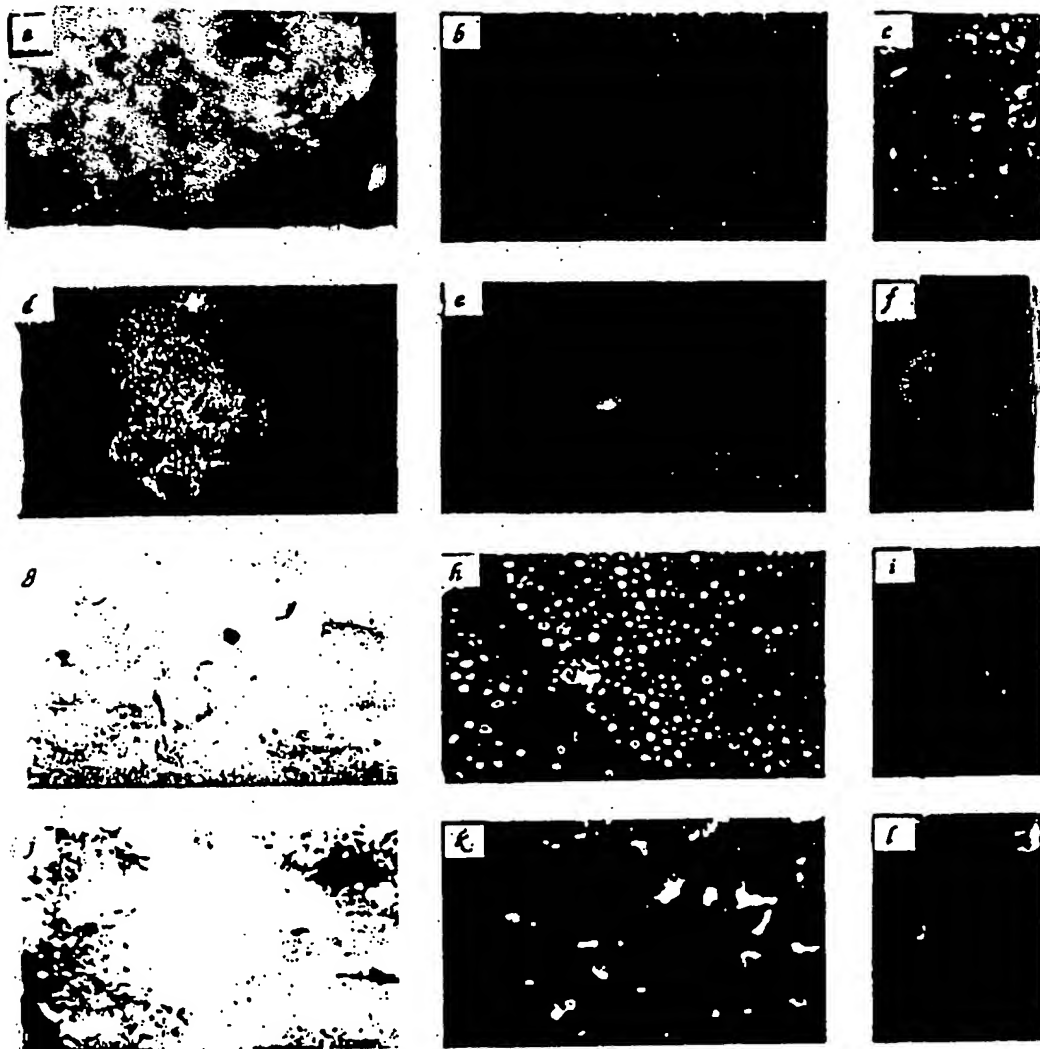
ANTIMALIGNIN ANTIBODY,
PICOGRAMS / CELL

FIGURE 5

Legend for Figure 5- Inhibition of growth of small cell lung carcinoma cells *in vitro* by antimalignin antibody. The inhibition is proportional to the concentration of antimalignin, which was 50% effective in the picogram per cell range. Each bar in the Figure represents the mean \pm SD for 24 wells, that is, from 3 wells for each of eight separate preparations of antimalignin at each dilution. **METHODS.** Small Cell Lung carcinoma cell line UCHNCU, grown in suspension and maintained in RPMI 1640 10% FCS (fetal calf serum) was seeded in 96 well microtitre plates (round bottom) at 10^4 cells per well. Serial dilutions were made of antimalignin antibody which had been purified by adsorption to immobilized malignin, original concentration 3 to 300 micrograms per ml, so that final concentration of antimalignin in RPMI FCS was 1/6 to 1/1458; final total volume per well was 200 microlitres; final concentration of antimalignin antibody 100 to 24,000 picograms per cell. Plates were incubated at 37°C in 6% CO₂ /air for 3 days. On day 3 cultures were pulsed with 1 μ ci/well tritiated thymidine (3HTdR) for 6 hours, then cultures were harvested with automatic cell harvester on filter pads. Filters were dried for 2 hours in 37°C dry incubator, discs were placed into scintillation vials, 2 ml Optiphase scintillant added, tubes capped, cpms counted on Beckman LS 1800 beta counter and % inhibition of cell growth calculated as Control minus Experimental/ Control x 100.

SUBSTITUTE SHEET (RULE 26)

6/6

**FIGURE 6**

Legend for Figure 6. Immunostaining of malignant cells with antimalignin antibody. a. squamous cell carcinoma of lung, cells obtained by bronchial washing; **b.** sclirous carcinoma of breast, fresh frozen section; **c.** retrobulbar malignant neuroectodermal tumor, fresh frozen section; **d.** carcinoma of pancreas, MIAPACA cell culture; **e.** lymphoma, single cell from pleural fluid; **f.** carcinoma of the vulva cells, frozen smear; **g.** small cell carcinoma of lung, alkaline phosphatase stain; **h.** acute lymphatic leukemia, two blast cells in blood smear, both fluorescent and natural light are on together; **i.** same slide as in h., but with natural light turned off and only fluorescent light on; **j., k., and l.** cytotoxicity of 50 microlitres of antimalignin antibody; 10 micrograms/ml, left in contact for varying periods of time with glioblastoma brain cancer cells growing on wall of tissue culture flask: **j.** 30 minutes at 5° C; **k.** 45 minutes at room temperature; **l.** one hour at room temperature, before the second layer for visualization was applied.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04553

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/138.1, 155.1, 156.1; 435/7.23; 436/94, 813; 514/14, 62, 391; 530/326, 327, 387.5 387.7; 536/23.5, 53.2; 930/DIG810

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GeneSeq, Swiss-Prot

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,840,915 (BOGOCH) 20 June 1989. See col. 1, line 30-col. 2, line 63; col. 4, lines 29-52; col. 5, lines 20-25; col. 5, line 65-col. 6, line 13; col. 24, line 6-col. 25, line 11; col. 28, line 60-col. 29, line 20.	2-3, 7-9, 13
X	US, A, 4,870,061 (SPECK) 26 September 1989. See col. 1, line 14-col. 2, line 34; col. 3, lines 4-30; col. 4, lines 61-68.	5-6
X	US, A, 5,220,008 (SABESAN) 15 June 1993. See col. 1, line 13-col. 2, line 12; col. 3, line 44-col. 4, line 2.	1

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* &	document member of the same patent family

Date of the actual completion of the international search

10 JULY 1995

Date of mailing of the international search report

11 AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DAVID SAUNDERS

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04553

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Cancer Detection and Prevention, Volume 17, Number 1, issued 1993, S. BOGOCH ET AL, "Malignin antibody returns to normal on successful treatment of breast cancer", page 180, abstract 276\35. See entire abstract.	3, 12
X	Journal of the National Cancer Institute, Volume 71, Number 2, issued August 1983, S. HAKOMORI ET AL, "Glycosphingolipids as tumor-associated and differentiation markers", pages 231-251. See page 233, col. 1; page 237, Table 6; page 238, col. 2-page 239, col. 2; page 246, cols. 1-2.	2-3
X	The Merck Index, 8th Edition, issued 1968, page 388, col. 2, entry entitled "Diphenylhydantoin". See entire entry.	5-6
X	Sigma Chemical Company (catalogue), "Biochemical Compounds for Research and Diagnostic Reagents", issued 1993. Page 459, col. 2. See product no. G-4875.	5-6
X	US, A, 4,918,170 (HASEGAWA ET AL.) 17 April 1990. See col. 1, lines 6-41.	7
X	American Journal of Psychiatry, Volume 123, Number 8, issued February 1967, R.J. CAMPBELL ET AL, "Cerebrospinal fluid glycoproteins in schizophrenia", pages 952-962. See page 952, col. 1; page 955, cols. 1-2; page 956, cols. 1-2 and page 957, Figure 1.	2, 4

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04553

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04553

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

A61K 31/41, 31/70, 38/10, 39/395; C07H 5/06, 21/02, 21/04; C07K 7/08, 16/18, 16/32; G01N 33/48, 33/574

A. CLASSIFICATION OF SUBJECT MATTER: US CL :

424/138.1, 155.1, 156.1; 435/7.23; 436/94, 813; 514/14, 62, 391; 530/326, 327, 387.5 387.7; 536/23.5, 53.2; 930/DIG810

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claim 1, drawn to glyco decoys (e.g. to prevent virus attachment to cells).
- II. Claim 2, drawn to methods of detection and production of aglyco products.
- III. Claims 2 and 12-13, drawn to antibodies to aglyco products (e.g. antimalignin) and their use in methods of diagnosis and treatment.
- IV. Claims 3-4, drawn to glycoconjugate products with reduced carbohydrate content.
- V. Claims 5-6, drawn to methods of treating schizophrenia.
- VI. Claims 7-8, drawn to therapeutic glycoconjugate products (e.g. 10B and its derivatives) for the improvement of memory.
- VII. Claim 8, drawn to DNA or RNA encoding 10B.
- VIII. Claims 9-11, drawn to peptide fragments of 10B related to cancer.

The glyco decoys used in Group I have a neuraminic acid-hexosamine linkage (disclosure page 2). They thus do not have their terminal neuraminic acid cleaved and are not aglyco products with reduced or altered carbohydrate content as are the aglyco products of the other Groups. These glyco decoy products thus do not share common structural feature with the products of the other Groups. The products of Group I are not novel; see review of art at disclosure pages 2-4.

Methods of detecting aglyco products in Group II are not novel. See review of art at disclosure pages 4-5 and applicant's own patents detecting malignin (e.g. 4,976,957 and 4,840,915).

The antibodies of Group III do not share a common structural core with the aglyco products recognized by these antibodies; the production and use of aglyco products and their antibodies, both recited in claim 2, must be recited in separate claims. It is thus proper for the ISA to presently place claim 2 in two Groups. The antibodies to aglyco products of Group III are not novel, e.g. antimalignin antibodies are known and the diagnosis of these in serum is known. See art reviewed at disclosure page 10 and applicant's patents (4,976,957 and 4,840,915).

The glycoconjugate products with reduced or altered carbohydrate content of Group IV are not novel. See art reviewed at disclosure pages 4-5.

Methods of treating schizophrenia of Group V are not novel. See art reviewed at disclosure page 5. The products used in these methods (e.g. hexoses) are not glycoconjugates and thus do not share a common structural feature with compositions recited in the other Groups. The glycoconjugate products (e.g. 10B and its derivatives) of Group VI are not novel. See art reviewed at disclosure page 9 and patents 4,976,957 and 4,840,915.

The DNA or RNA of Group VII has no common core structure with the encoded 10B products. Nucleic acids and their encoded products cannot be claimed in the same claim. It is thus proper for the ISA to presently place claim 8 in two Groups.

The peptide fragments of Group VIII do not share common structural features with the glyco decoys of Group I, the aglyco products of reduced/altered carbohydrate content of Groups II, IV, V and VI, the antibodies of Group III, or the

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04553

nucleic acid products of Group VII. The peptide fragments of Group VIII are not novel; see art reviewed at disclosure page 9 and patents 4,976,957 and 4,840,915.

Note that lack of novelty noted in the above Groups is an indication that the inventions lack a "special technical feature" which defines the contribution which each makes over the prior art. For this reason and for the above noted lack of common structural features among the compositions of the various Groups, unity of invention is lacking.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.